

# **METHODS FOR MAKING PLANTS TOLERANT TO GLYPHOSATE AND COMPOSITIONS THEREOF**

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genetic engineering for herbicide resistance and, more particularly, to a novel glyphosate  
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enolpyruvylshikimate-3-phosphate synthase gene isolated and purified from *Eleusine*  
15 *indica* into crop and ornamental plants of economic importance.

## **BACKGROUND OF THE INVENTION**

N-phosphonomethylglycine, also known as glyphosate, is a well known  
herbicide that has activity on a broad spectrum of plant species. Glyphosate is the active  
20 ingredient of Roundup® (Monsanto Co.), a safe herbicide having a desirably short half life  
in the environment. When applied onto a plant surface, glyphosate moves systemically  
through the plant. Glyphosate is toxic to plants by inhibiting the shikimic acid pathway  
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This invention relates in general to plant molecular biology and plant genetic engineering for herbicide resistance and, more particularly, to a novel glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase from *Eleusine indica*. Plant genetic engineering methods can be used to transfer the glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase gene isolated and purified from *Eleusine indica* into crop and ornamental plants of economic importance.

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## BACKGROUND OF THE INVENTION

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N-phosphonomethylglycine, also known as glyphosate, is a well known herbicide that has activity on a broad spectrum of plant species. Glyphosate is the active ingredient of Roundup® (Monsanto Co.), a safe herbicide having a desirably short half life in the environment. When applied onto a plant surface, glyphosate moves systemically through the plant. Glyphosate is toxic to plants by inhibiting the shikimic acid pathway that provides a precursor for the synthesis of aromatic amino acids. Specifically, glyphosate affects the conversion of phosphoenolpyruvate and 3-phosphoshikimic acid to

5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimate synthase (hereinafter referred to as EPSP synthase or EPSPS). For purposes of the present invention, the term “glyphosate” should be considered to include any herbicidally effective form of N-phosphonomethylglycine (including any salt thereof) and other forms that result in the production of the glyphosate anion in planta.

Through plant genetic engineering methods, it is possible to produce glyphosate tolerant plants by inserting into the plant genome a DNA molecule that causes the production of higher levels of wild-type EPSPS (Shah *et al.*, Science 233:478-481 (1986). Glyphosate tolerance can also be achieved by the expression of EPSPS variants that have lower affinity for glyphosate and therefore retain their catalytic activity in the presence of glyphosate (U.S. Patent No. 4,940,835, U.S. Patent No. 5,094,945, U.S. Patent No. 5,633,435). Enzymes that degrade glyphosate in the plant tissues (U.S. Patent No. 5,463,175) are also capable of conferring cellular tolerance to glyphosate. Such genes, therefore, allow for the production of transgenic crops that are tolerant to glyphosate, thereby allowing glyphosate to be used for effective weed control with minimal concern of crop damage. For example, glyphosate tolerance has been genetically engineered into corn (U.S. Patent No. 5,554,798), wheat (Zhou *et al.* Plant Cell Rep. 15:159-163 (1995), soybean (WO 9200377) and canola (WO 9204449).

Variants of the wild-type EPSPS enzyme are glyphosate-resistant as a result of alterations in the EPSPS amino acid coding sequence (Kishore *et al.*, Annu. Rev. Biochem. 57:627-663 (1988); Schulz *et al.*, Arch. Microbiol. 137:121-123 (1984); Sost *et al.*, FEBS Lett. 173:238-241 (1984); Kishore *et al.*, In “Biotechnology for Crop Protection” ACS Symposium Series No. 379. Eds. Hedlin *et al.*, 37-48 (1988). These

variants typically have a higher  $K_i$  for glyphosate than the wild-type EPSPS enzyme that confers the glyphosate-tolerant phenotype, but these variants are also characterized by a high  $K_m$  for PEP that makes the enzyme kinetically less efficient. For example, the apparent  $K_m$  for PEP and the apparent  $K_i$  for glyphosate for the native EPSPS from *E. coli* are 10  $\mu$ M and 0.5  $\mu$ M while for a glyphosate-resistant isolate having a single amino acid substitution of an alanine for the glycine at position 96 these values are 220  $\mu$ M and 4.0 mM, respectively. A number of glyphosate-resistant plant variant EPSPS genes have been constructed by mutagenesis.

A variety of native and variant EPSPS enzymes have been expressed in transgenic plants in order to confer glyphosate tolerance (Singh, *et al.*, In "Biosynthesis and Molecular Regulation of Amino Acids in Plants", Amer Soc Plant Phys. Pubs (1992). Examples of some of these EPSP Synthases and methods for preparing transgenic plants resistant to glyphosate include those described and/or isolated in accordance with U.S. Patent No. 4,940,835, U.S. Patent No. 4,971,908, U.S. Patent No. 5,145,783, U.S. Patent No. 5,188,642, U.S. Patent No. 5,310,667, U.S. Patent No. 5,312,910, and U.S. Patent No. 6,40,497. They can also be derived from a structurally distinct class of non-homologous EPSPS genes, such as the naturally occurring class II EPSPS genes isolated from *Agrobacterium* sp. strain CP4 as described in U.S. Patent No. 5,633,435 and U.S. Patent No. 5,627,061.

*Eleusine indica* is commonly referred to as "goose grass" and may also be known as "yard grass". It is a common monocotyledonous plant found world wide. As a member of the Poaceae family, the grass family, it is related to many well known crop plants.

*Eleusine indica* is most closely related to the millets, that include *Sorghum bicolor*

(sorghum or great millet), *Zea mays* (maize), *Pennisetum americanum* (pearl millet),  
*Eleusine coracana* (finger millet), *Setaria italica* (foxtail millet), *Paspalum scrobiculatum*  
(kodo millet), *Echinochloa frumentacea* (barnyard millet) and *Eragrostis tef* (teff)  
(Chennaveeraiah *et al.*, In "Chromosome engineering in plants: genetics, breeding and  
5 evolution", Cytogenetics of Minor Millets, in Tsuchiya *et al.*, eds Elsevier Sci Pub  
Amsterdam, 613-627 (1991). *Eleusine indica* has been shown to hybridize with *Eleusine*  
*coracana* (finger millet), an important cultivated millet of India and East Africa  
(Chennaveeraiah *et al.*, Euphytica 2-3:489-495, (1974). Classical plant breeding methods  
can be used to transfer the genes and traits of interest from *Eleusine indica* into  
10 agronomic crop plants within the family Poaceae.

### SUMMARY OF THE INVENTION

In its broadest sense, the present invention herein provides a method for plant  
tolerance to glyphosate herbicide by the expression of an isolated DNA molecule encoding  
15 a naturally occurring glyphosate resistant EPSPS enzyme. The enzyme and the DNA is  
isolated from *Eleusine* species, more particularly *Eleusine indica* (*E. indica*).

In the first aspect of the present invention described herein provides a method to  
cause plants to be tolerant to glyphosate herbicide by the insertion of a recombinant DNA  
molecule into the nuclear genome of a plant cell, the recombinant DNA molecule  
20 comprising:

a promoter that functions in plant cells to cause the production of an RNA  
molecule; operably linked to,

a DNA molecule transcribing an RNA encoding for a chloroplast transit peptide and a *E. indica* glyphosate resistant EPSPS enzyme; operably linked to,

a 3' non-translated region that functions in plant cells to cause the polyadenylation of the 3' end of the RNA molecule.

5 Typically, the promoter used in the DNA molecule is expressed in a constitutive fashion. Examples of suitable promoters that function effectively in this capacity include cauliflower mosaic virus 19S promoter, cauliflower mosaic virus 35S promoter, figwort mosaic virus 34S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, small subunit of ribulose-1,5-bisphosphate carboxylase promoter,  
10 rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferase promoter, rice actin 1 promoter, maize ubiquitin promoter, mannopine synthase promoter and octopine synthase promoter. A Promoter may also comprise leader sequences and intron sequences useful in the invention.

A DNA molecule that encodes a chloroplast transit peptide sequence can be  
15 isolated from EPSPS genes purified from various plant species including *E. indica* as well as from various plant genes whose protein products have been shown to be transported into the chloroplast.

A DNA molecule that encodes a glyphosate resistant EPSPS enzyme isolated from *Eleusine* species, more particularly from *E. indica*, comprising SEQ ID NO:7 is an object  
20 of the invention and a DNA molecule substantially homologous to the DNA molecule isolated from *E. indica* or a portion thereof identified as SEQ ID NO:6.

The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated, the 3' untranslated region from pea

small subunit Rubisco gene, the wheat heat shock protein 17.9 3' untranslated region, the 3' untranslated region from soybean 7S seed storage protein gene are commonly used in this capacity.

The invention also relates to a glyphosate tolerant transgenic crop plant cell, a  
5 glyphosate tolerant crop plant and crop plant parts, crop seeds and progeny thereof comprising the recombinant DNA molecule of the present invention.

A DNA molecule that encodes a naturally occurring plant derived glyphosate resistant EPSPS enzyme, wherein the glyphosate resistant EPSPS enzyme has a  $K_m$  for phosphoenolpyruvate (PEP) of less than 10  $\mu$ M. More preferably, a DNA molecule that  
10 encodes a naturally occurring plant derived glyphosate resistant EPSPS enzyme wherein the glyphosate resistant EPSPS enzyme has a  $K_m$  for PEP of less than 10  $\mu$ M and the  $K_m$  for PEP is not more than about 2 X of the naturally occurring plant derived glyphosate sensitive EPSPS enzyme.

A DNA molecule that encodes a naturally occurring glyphosate resistant EPSPS  
15 enzyme derived from *Eleusine* species, wherein the glyphosate resistant EPSPS enzyme has a  $K_m$  for phosphoenolpyruvate (PEP) of less than 10  $\mu$ M. More preferably, a DNA molecule that encodes a naturally occurring glyphosate resistant EPSPS enzyme derived from *Eleusine* species, wherein the glyphosate resistant EPSPS enzyme has a  $K_m$  for PEP of less than 10  $\mu$ M and the  $K_m$  for PEP is not more than about 2 X of the naturally  
20 occurring plant derived glyphosate sensitive EPSPS enzyme.

A DNA molecule that encodes a naturally occurring glyphosate resistant EPSPS enzyme derived from *E. indica*, wherein the naturally occurring glyphosate resistant EPSPS enzyme amino acid sequence has been modified by amino acid substitutions

selected from the group consisting of threonine to isoleucine at amino acid position 103 and glycine to alanine at amino acid position 102.

The invention also relates to the homologous genetic elements regulating expression of the *E. indica* glyphosate resistant EPSPS gene. These elements include but are not limited to the DNA sequences of a promoter, a 5' untranslated region, a chloroplast transit peptide, an intron, and a 3' untranslated region of *E. indica* EPSPS glyphosate resistance gene. A DNA molecule that encodes a glyphosate resistant EPSPS enzyme purified from the genome of *Eleusine* species, more particularly from *E. indica* glyphosate resistant biotype provided by the ATCC deposit # PTA-2177 is an object of the invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1. *Eleusine indica* (glyphosate tolerant) EPSP synthase DNA sequence (SEQ ID NO:6).

Figure 2. Deduced amino acid sequence for the mature protein-coding region of the *Eleusine indica* (glyphosate tolerant biotype) EPSP synthase gene (SEQ ID NO:7).



Figure 3. Growth rates on glyphosate containing media of transgenic *E. coli* on expressing the *E. indica* glyphosate sensitive EPSPS enzyme and the *E. indica* glyphosate resistant EPSPS enzyme.

Figure 4. Glyphosate inhibition study comparing the EPSP synthase activities detectable in extracts prepared from the glyphosate-sensitive and tolerant *E. indica* biotypes.

Figure 5. Plasmid map of pMON45364

Figure 6. Plasmid map of pMON45365

Figure 7. Plasmid map of pMON45367

Figure 8. Plasmid map of pMON45369

Figure 9. The amino acid sequence deduced from the cDNA sequence of the mature EPSP synthase protein sequence derived from the glyphosate-tolerant *E. indica* biotype (top row) aligned with that of the glyphosate sensitive *E. indica* biotype (bottom row).

## DETAILED DESCRIPTION OF THE INVENTION

This application claims the benefit of U.S. Provisional Application No. 60/188,093, filed March 9, 2000.

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger *et al.*, Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York, (1991); and Lewin, Genes V, Oxford

University Press: New York, (1994). The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used. The standard one- and three-letter nomenclature for amino acid residues is used.

“cDNA library” refers to a collection of cDNA fragments, each cloned into a  
5 separate vector molecule.

The term “chimeric” refers to a fusion nucleic acid or protein sequence. A chimeric nucleic acid coding sequence is comprised of two or more sequences joined in-frame that encode a chimeric protein. A chimeric gene refers to the multiple genetic elements derived from heterologous sources comprising a gene.

10 The phrases “coding sequence”, “open reading frame”, and “structural sequence” refer to the region of continuous sequential nucleic acid triplets encoding a protein, polypeptide, or peptide sequence.

“Codon” refers to a sequence of three nucleotides that specify a particular amino acid.

15 “Complementarity” and “complement” when referring to nucleic acid sequences, refers to the specific binding of adenine to thymine (or uracil in RNA) and cytosine to guanine on opposite strands of DNA or RNA.

“Construct” refers to the heterologous genetic elements operably linked to each other making up a recombinant DNA molecule.

20 “C-terminal region” refers to the region of a peptide, polypeptide, or protein chain from the middle thereof to the end that carries the amino acid having a free carboxyl group.

The term “encoding DNA” refers to chromosomal DNA, plasmid DNA, cDNA, or synthetic DNA that encodes any of the proteins discussed herein.

The term “endogenous” refers to materials originating from within an organism or cell.

5        “Endonuclease” refers to an enzyme that hydrolyzes double stranded DNA at internal locations.

“Exogenous” refers to materials originating from outside of an organism or cell. This typically applies to nucleic acid molecules used in producing transformed or transgenic host cells and plants.

10        “Exon” refers to the portion of a gene that is actually translated into protein, i.e. a coding sequence.

The term “expression” refers to the transcription of a gene to produce the corresponding mRNA.

“Fragments”. A fragment of a EPSPS gene is a portion of a full-length EPSPS  
15        gene nucleic acid that is of at least a minimum length capable of expressing a protein with EPSPS activity.

The term “gene” refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

20        The term “genome” as it applies to viruses encompasses all of the nucleic acid sequence contained within the capsid of the virus. The term “genome” as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. Encoding nucleic acids of the present invention introduced into bacterial host cells can

therefore be either chromosomally-integrated or plasmid-localized. The term “genome” as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components of the cell. Nucleic acids of the present invention introduced into plant cells can therefore be either

5 chromosomally-integrated or organelle-localized.

“Glyphosate” refers to N-phosphonomethylglycine and its’ salts, Glyphosate is the active ingredient of Roundup® herbicide (Monsanto Co.). Plant treatments with “glyphosate” refer to treatments with the Roundup® or Roundup Ultra® herbicide formulation, unless otherwise stated. Glyphosate as N-phosphonomethylglycine and its’

10 salts (not formulated Roundup® herbicide) are components of synthetic culture media used for the selection of bacteria and plant tolerance to glyphosate or used to determine enzyme resistance in in vitro biochemical assays.

“Heterologous DNA” refers to DNA from a source different than that of the recipient cell.

15 “Homologous DNA” refers to DNA from the same source as that of the recipient cell.

“Hybridization” refers to the ability of a strand of nucleic acid to join with a complementary strand via base pairing. Hybridization occurs when complementary sequences in the two nucleic acid strands bind to one another.

20 “Identity” refers to the degree of similarity between two nucleic acid or protein sequences. An alignment of the two sequences is performed by a suitable computer program. A widely used and accepted computer program for performing sequence alignments is CLUSTALW v1.6 (Thompson, *et al.* Nucl. Acids Res., 22: 4673-4680,

1994). The number of matching bases or amino acids is divided by the total number of bases or amino acids, and multiplied by 100 to obtain a percent identity. For example, if two 580 base pair sequences had 145 matched bases, they would be 25 percent identical. If the two compared sequences are of different lengths, the number of matches is divided  
 5 by the shorter of the two lengths. For example, if there are 100 matched amino acids between 200 and a 400 amino acid proteins, they are 50 percent identical with respect to the shorter sequence. If the shorter sequence is less than 150 bases or 50 amino acids in length, the number of matches are divided by 150 (for nucleic acid bases) or 50 (for amino acids), and multiplied by 100 to obtain a percent identity.

10        "Intron" refers to a portion of a gene not translated into protein, even though it is transcribed into RNA.

"Isolated" An "isolated" nucleic acid is one that has been substantially separated or purified away from other nucleic acid sequences in the cell of the organism that the nucleic acid naturally occurs, i.e., other chromosomal and extrachromosomal DNA and  
 15 RNA, by conventional nucleic acid-purification methods. The term also embraces recombinant nucleic acids and chemically synthesized nucleic acids.

"Native" The term "native" refers to a naturally-occurring ("wild-type") nucleic acid or polypeptide.

"N-terminal region" refers to the region of a peptide, polypeptide, or protein chain  
 20 from the amino acid having a free amino group to the middle of the chain.

"Nucleic acid" refers to deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

Nucleic acid codes: A = adenosine; C = cytosine; G = guanosine; T = thymidine.

Codes used for synthesis of oligonucleotides: N = equimolar A, C, G, and T; I =

deoxyinosine; K = equimolar G and T; R = equimolar A and G; S = equimolar C and G; W = equimolar A and T; Y = equimolar C and T.

A "nucleic acid segment" or a "nucleic acid molecule segment" is a nucleic acid molecule that has been isolated free of total genomic DNA of a particular species, or that  
 5 has been synthesized. Included with the term "nucleic acid segment" are DNA segments, recombinant vectors, plasmids, cosmids, phagemids, phage, viruses, et cetera.

"Nucleic-Acid Hybridization"; "Stringent Conditions"; "Specific" The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by  
 10 the specific hybridization procedure discussed in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989), at 9.52-9.55. See also, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989) at 9.47-9.52, 9.56-9.58; Kanehisa, *Nucl. Acids Res.* 12:203-213, (1984); and Wetmur and Davidson, *J. Mol. Biol.* 31:349-370, (1968).

15 "Nucleotide Sequence Variants" Using well-known methods, the skilled artisan can readily produce nucleotide and amino acid sequence variants of EPSPS genes and proteins, respectively. "Variant" DNA molecules are DNA molecules containing minor changes in a native EPSPS gene sequence, i.e., changes that one or more nucleotides of a native EPSPS gene sequence is deleted, added, and/or substituted, such that the variant  
 20 EPSPS gene encodes a protein that retains EPSPS activity. Variant DNA molecules can be produced, for example, by standard DNA mutagenesis techniques or by chemically synthesizing the variant DNA molecule or a portion thereof. Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage *et al.*, *Tetra. Letts.*

22:1859-1862 (1981), and Matteucci *et al.*, J. Am. Chem. Soc. 103:3185- (1981).

Chemical synthesis of nucleic acids can be performed, for example, on automated oligonucleotide synthesizers. Such variants preferably do not change the reading frame of the protein-coding region of the nucleic acid and preferably encode a protein having no amino acid changes. Nucleic acid sequence variants are most often created for the purposes of modification of the sequence to add or delete restriction endonuclease sites or to affect transcription or translation of the nucleic acid molecule.

“Amino-acid substitutions”, “Amino-acid variants”, are preferably substitutions of single amino-acid residue for another amino-acid residue at any position within the protein. Substitutions, deletions, insertions or any combination thereof can be combined to arrive at a final construct.

“Open reading frame (ORF)” refers to a region of DNA or RNA encoding a peptide, polypeptide, or protein.

“Operably Linked”. A first nucleic-acid sequence is “operably” linked with a second nucleic-acid sequence when the first nucleic-acid sequence is placed in a functional relationship with the second nucleic-acid sequence. For instance, a promoter is operably linked to a protein-coding sequence if the promoter effects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in reading frame.

“Overexpression” refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said

host cell at a higher level than that normally expressed from the endogenous gene encoding said polypeptide or protein.

“Plant expression vector” refers to chimeric DNA molecules comprising the regulatory elements that are operably linked to provide the expression of a transgene product in plants.

“Plasmid” refers to a circular, extrachromosomal, self-replicating piece of DNA.

“Polyadenylation signal” or “polyA signal” refers to a nucleic acid sequence located 3’ to a coding region that causes the addition of adenylate nucleotides to the 3’ end of the mRNA transcribed from the coding region.

“Polymerase chain reaction (PCR)” refers to an enzymatic technique to create multiple copies of one sequence of nucleic acid. Copies of DNA sequence are prepared by shuttling a DNA polymerase between two amplimers. The basis of this amplification method is multiple cycles of temperature changes to denature, then re-anneal amplimers, followed by extension to synthesize new DNA strands in the region located between the flanking amplimers.

The term “promoter” or “promoter region” refers to a nucleic acid sequence, usually found upstream (5’) to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site. As contemplated herein, a promoter or promoter region includes variations of promoters derived by means of ligation to various regulatory sequences, random or controlled mutagenesis, and addition or duplication of enhancer sequences. The promoter region disclosed herein, and biologically functional equivalents



thereof, are responsible for driving the transcription of coding sequences under their control when introduced into a host as part of a suitable recombinant vector, as demonstrated by its ability to produce mRNA.

"Recombinant". A "recombinant" nucleic acid is made by an artificial combination  
5 of two otherwise separated segments of sequence, *e.g.*, by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The term "recombinant DNA construct" or "recombinant vector" refers to any agent such as a plasmid, cosmid, virus, autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence,  
10 derived from any source, capable of genomic integration or autonomous replication, comprising a DNA molecule that one or more DNA sequences have been linked in a functionally operative manner. Such recombinant DNA constructs or vectors are capable of introducing a 5' regulatory sequence or promoter region and a DNA sequence for a selected gene product into a cell in such a manner that the DNA sequence is transcribed  
15 into a functional mRNA that is translated and therefore expressed. Recombinant DNA constructs or recombinant vectors may be constructed to be capable of expressing antisense RNAs, in order to inhibit translation of a specific RNA of interest.

"Regeneration" refers to the process of growing a plant from a plant cell (*e.g.*, plant protoplast or explant).

20 "Reporter" refers to a gene and corresponding gene product that when expressed in transgenic organisms produces a product detectable by chemical or molecular methods or produces an observable phenotype.

“Restriction enzyme” refers to an enzyme that recognizes a specific palindromic sequence of nucleotides in double stranded DNA and cleaves both strands; also called a restriction endonuclease. Cleavage typically occurs within the restriction site.

“Selectable marker” refers to a nucleic acid sequence whose expression confers a phenotype facilitating identification of cells containing the nucleic acid sequence.

Selectable markers include those that confer resistance to toxic chemicals (e.g. ampicillin resistance, kanamycin resistance), complement a nutritional deficiency (e.g. uracil, histidine, leucine), or impart a visually distinguishing characteristic (e.g. color changes or fluorescence). Useful dominant selectable marker genes include genes encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin); and herbicide resistance genes (e.g., phosphinothricin acetyltransferase). A useful strategy for selection of transformants for herbicide resistance is described, e.g., in Vasil, Cell Culture and Somatic Cell Genetics of Plants, Vols. I-III, Laboratory Procedures and Their Applications Academic Press, New York (1984).

The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under given hybridization conditions only to the target sequence in a sample comprising the target sequence.

“Tolerant” refers to a reduced toxic effect of glyphosate on the growth and development of microorganisms and plants.

“Transcription” refers to the process of producing an RNA copy from a DNA template.

“Transformation” refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, recombinant nucleic acid molecule) into a cell or protoplast that

exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication.

"Transformed" or "transgenic" refers to a cell, tissue, organ, or organism into that has been introduced a foreign nucleic acid, such as a recombinant vector. A "transgenic" or "transformed" cell or organism also includes progeny of the cell or organism and progeny produced from a breeding program employing such a "transgenic" plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of the foreign nucleic acid.

The term "transgene" refers to any nucleic acid sequence nonnative to a cell or organism transformed into said cell or organism. "Transgene" also encompasses the component parts of a native plant gene modified by insertion of a nonnative nucleic acid sequence by directed recombination.

The term "translation" refers to the production the corresponding gene product, i.e., a peptide, polypeptide, or protein from a mRNA.

"Vector" refers to a plasmid, cosmid, bacteriophage, or virus that carries foreign DNA into a host organism.

"Isolated," "Purified," "Homogeneous" Polypeptides. A polypeptide is "isolated" if it has been separated from the cellular components (nucleic acids, lipids, carbohydrates, and other polypeptides) that naturally accompany it or that is chemically synthesized or recombinant. A monomeric polypeptide is isolated when at least 60% by weight of a sample is composed of the polypeptide, preferably 90% or more, more preferably 95% or more, and most preferably more than 99%. Protein purity or homogeneity is indicated, for example, by polyacrylamide gel electrophoresis of a protein sample, followed by

visualization of a single polypeptide band upon staining the polyacrylamide gel; high pressure liquid chromatography; or other conventional methods. Coat proteins can be purified by any of the means known in the art, for example as described in Guide to Protein Purification, ed. Deutscher, Meth. Enzymol. 185, Academic Press, San Diego, 5 1990; and Scopes, Protein Purification: Principles and Practice, Springer Verlag, New York, 1982.

“Labeling”. There are a variety of conventional methods and reagents for labeling polypeptides and fragments thereof. Typical labels include radioactive isotopes, ligands or ligand receptors, fluorophores, chemiluminescent agents, and enzymes. Methods for 10 labeling and guidance in the choice of labels appropriate for various purposes are discussed, *e.g.*, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989) and Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, (1992).

“Mature protein coding region”. This term refers to the sequence of the processed 15 protein product of EPSPS remaining after the chloroplast transit peptide sequence has been removed.

“Polypeptide fragments”. The present invention also encompasses fragments of an *E indica* EPSPS that lacks at least one residue of a native full-length *E. indica* EPSPS protein, but that specifically maintains EPSPS activity.

20 “Transit peptide or targeting peptide sequence”. These terms generally refer to peptide sequences that when linked to a protein of interest directs the protein to a particular tissue, cell, subcellular location, or cell organelle. Examples include, but are not limited to, chloroplast transit peptides, nuclear targeting signals, and vacuolar signals. The

chloroplast transit peptide is of particular utility in the present invention to direct expression of the EPSPS enzyme to the chloroplast.

The term "plant" encompasses any higher plant and progeny thereof, including monocots (*e.g.*, corn, rice, wheat, barley, *etc.*), dicots (*e.g.*, soybean, cotton, tomato, 5 potato, *Arabidopsis*, tobacco, *etc.*), gymnosperms (pines, firs, cedars, etc) and includes parts of plants, including reproductive units of a plant (*e.g.*, seeds, bulbs, tubers, or other parts or tissues from that the plant can be reproduced), fruits and flowers.

Exogenous genetic material may be transferred into a plant by the use of a DNA vector designed for such a purpose by methods that utilize *Agrobacterium*, particle 10 bombardment or other methods known to those skilled in the art. A particularly preferred subgroup of exogenous material comprises a nucleic acid molecule of the present invention. Design of such a vector is generally within the skill of the art (Plant Molecular Biology: A Laboratory Manual, eds. Clark, Springer, New York (1997). Examples of such plants in to which exogenous genetic material may be transferred, include, without 15 limitation, alfalfa, *Arabidopsis*, barley, Brassica, broccoli, cabbage, citrus, cotton, garlic, oat, oilseed rape, onion, canola, flax, maize, an ornamental annual and ornamental perennial plant, pea, peanut, pepper, potato, rice, rye, sorghum, soybean, strawberry, sugarcane, sugar beet, tomato, wheat, poplar, pine, fir, eucalyptus, apple, lettuce, lentils, grape, banana, tea, turf grasses, sunflower, oil palm, *Phaseolus* etc.

20 The particular promoters selected for use in embodiments of the present invention should be capable of causing the production of sufficient expression to, in the case of the DNA molecule, generate protein expression in vegetative and reproductive tissues of a transformed plant. The DNA molecule will typically contain a constitutive promoter, a

structural DNA sequence encoding a herbicide resistant enzyme, and a 3' non-translated region. A number of constitutive promoters that are active in plant cells have been described. Suitable promoters for constitutive expression in plants of herbicide tolerance for the DNA molecule include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al.* Nature 313:801-812 (1985), the Figwort mosaic virus (FMV) 35S (Sanger *et al.* Plant Mol. Biol. 14:433-443 (1990), the sugarcane bacilliform virus promoter (Bouhida *et al.*, J. Gen. Virol. 74:15-22 (1993), the commelina yellow mottle virus promoter (Medberry *et al.*, Plant J. 3:619-626 (1993), the light-inducible promoter from the small subunit of the ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO) (Coruzzi *et al.*, EMBO J. 3:1671-1679 (1984), the rice cytosolic triosephosphate isomerase (TPI) promoter (Xu *et al.* Plant Physiol. 106:459-467 (1994), the adenine phosphoribosyltransferase (APRT) promoter of *Arabidopsis* (Moffatt *et al.* Gene 143:211-216 (1994), the rice actin 1 gene promoter (Zhong *et al.* Plant Sci. 116:73-84 (1996), the mannopine synthase and octopine synthase promoters (Ni *et al.* Plant J. 7:661-676 (1995), the Adh promoter (Walker *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:6624-6628 (1987), the sucrose synthase promoter (Yang *et al.*, Proc. Natl. Acad. Sci. U.S.A. 87: 4144-4148 (1990), the R gene complex promoter (Chandler *et al.*, The Plant Cell 1:1175-1183 (1989), and the chlorophyll  $\alpha/\beta$  binding protein gene promoter, et cetera. These promoters have been used to create DNA vectors that have been expressed in plants; *see, e.g.*, PCT publication WO 8402913. All of these promoters have been used to create various types of plant-expressible recombinant DNA vectors. Comparative analysis of constitutive promoters by the expression of reporter genes such as the *uidA* ( $\beta$ -glucuronidase) gene from *E. coli* has been performed with many of these and other

promoters ( Li *et al.* Mol. Breeding 3:1-14 (1997); Wen *et al.* Chinese J. of Bot. 5:102-109 (1993). Promoters that are known or are found to cause transcription of DNA in plant cells can be used in the present invention. Such promoters may be obtained from a variety of sources such as plants and plant viruses. In addition to promoters that are

5 known to cause transcription of DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes that are selectively or preferably expressed in the target tissues or cells. For the purpose of expression in source tissues of the plant, such as the leaf, seed, root or stem, it is preferred that the promoters utilized in the present invention have relatively high expression in these

10 specific tissues. For this purpose, one may choose from a number of promoters for genes with tissue- or cell-specific or -enhanced expression. Examples of such promoters reported in the literature include the chloroplast glutamine synthetase GS2 promoter from pea (Edwards *et al.*, Proc. Natl. Acad. Sci. U.S.A. 87: 3459-3463 (1990), the chloroplast fructose-1,6-biphosphatase (FBPase) promoter from wheat (Lloyd *et al.*, Mol. Gen.

15 Genet. 225: 209-216 (1991), the nuclear photosynthetic ST-LS1 promoter from potato (Stockhaus *et al.*, EMBO J. 8: 2445-2451, (1989), the serine/threonine kinase (PAL) promoter and the glucoamylase (CHS) promoter from Arabidopsis thaliana. Also reported to be active in photosynthetically active tissues are the ribulose-1,5-bisphosphate carboxylase (RBCS) promoter from eastern larch (*Larix laricina*), the promoter for the

20 Cab gene, Cab6, from pine (Yamamoto *et al.*, Plant Cell Physiol. 35: 773-778 (1994), the promoter for the Cab-1 gene from wheat (Fejes *et al.*, Plant Mol. Biol. 15: 921-932 (1990), the promoter for the Cab-1 gene from spinach (Lubberstedt *et al.*, Plant Physiol. 104:997-1006 (1994), the promoter for the Cab1R gene from rice (Luan *et al.*, Plant Cell.

4:971-981 (1992), the pyruvate, orthophosphate dikinase (PPDK) promoter from *Zea mays* (Matsuoka *et al.*, Proc. Natl. Acad. Sci. U.S.A. 90: 9586-9590 (1993), the promoter for the tobacco Lhcb1\*2 gene (Cerdan *et al.*, Plant Mol. Biol. 33:245-255 (1997), the *Arabidopsis thaliana* Suc2 sucrose-H<sup>+</sup> symporter promoter (Truernit *et al.*, Planta. 196:564-570 (1995), and the promoter for the thylakoid membrane protein genes from spinach (PsaD, PsaF, PsaE, PC, FNR, AtpC, AtpD, Cab, RbcS).

Other promoters for the chlorophyll  $\alpha/\beta$ -binding proteins may also be utilized in the present invention, such as the promoters for Lhcb gene and PsbP gene from white mustard (*Sinapis alba*) (Kretsch *et al.*, Plant Mol. Biol. 28: 219-229 (1995). A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of RNA-binding protein genes in plant cells, including promoters regulated by (1) heat (Callis *et al.*, Plant Physiol. 88:965-968 (1988), (2) light (e.g., pea RbcS-3A promoter, Kuhlemeier *et al.*, Plant Cell 1:471-478 (1989); maize RbcS promoter, Schaffner *et al.*, Plant Cell 3:997-1012 (1991); (3) hormones, such as abscisic acid (Marcotte *et al.*, Plant Cell 1:969-976 (1989), (4) wounding (e.g., WunI, Siebertz *et al.*, Plant Cell 1:961-968 (1989); or (5) chemicals, such as methyl jasminate, salicylic acid, steroid hormones, alcohol, Safeners (Gatz, Curr. Opin. Biotech 7:168-172 (1996), WO 9706269), or it may also be advantageous to employ (6) organ-specific promoters (e.g., Roshal *et al.*, EMBO J. 6:1155- (1987); Scherthaner *et al.*, EMBO J. 7:1249-1255 (1988); Bustos *et al.*, Plant Cell 1:839-853 (1989).

For the purpose of expression in sink tissues of the plant, such as the tuber of the potato plant, the fruit of tomato, or the seed of soybean, canola, cotton, *Zea mays*, wheat, rice, and barley, it is preferred that the promoters utilized in the present invention have



relatively high expression in these specific tissues. A number of promoters for genes with tuber-specific or -enhanced expression are known, including the class I patatin promoter (Bevan *et al.*, EMBO J. 8:1899-1906 (1986); Jefferson *et al.*, Plant Mol. Biol. 14:995-1006 (1990), the promoter for the potato tuber ADPGPP genes, both the large and small subunits, the sucrose synthase promoter (Salanoubat *et al.*, Gene 60:47-56 (1987); Salanoubat *et al.*, Gene 84:181-185 (1989), the promoter for the major tuber proteins including the 22 kD protein complexes and proteinase inhibitors (Hannapel, Plant Physiol. 101:703-704 (1993), the promoter for the granule bound starch synthase gene (GBSS) (Visser *et al.*, Plant Mol. Biol. 17:691-699 (1991), and other class I and II patatins promoters (Koster-Topfer *et al.*, Mol. Gen. Genet. 219:390-396 (1989); Mignery *et al.*, Gene 62:27-44 (1988). Other promoters can also be used to express a protein in specific tissues, such as seeds or fruits. The promoter for  $\beta$ -conglycinin (Chen *et al.*, Dev. Genet. 10:112-122 (1989) or other seed-specific promoters such as the napin and phaseolin promoters, can be used. The zeins are a group of storage proteins found in *Zea mays* endosperm. Genomic clones for zein genes have been isolated (Pedersen *et al.*, Cell 29:1015-1026 (1982), and the promoters from these clones, including the 15 kD, 16 kD, 19 kD, 22 kD, 27 kD, and gamma genes, could also be used. Other promoters known to function, for example, in *Zea mays* include the promoters for the following genes: *waxy*, *Brittle*, *Shrunken 2*, Branching enzymes I and II, starch synthases, debranching enzymes, oleosins, glutelins, and sucrose synthases. A particularly preferred promoter for *Zea mays* endosperm expression is the promoter for the glutelin gene from rice, more particularly the Osgt-1 promoter (Zheng *et al.*, Mol. Cell Biol. 13:5829-5842 (1993). Examples of promoters suitable for expression in wheat include those promoters for the ADPglucose

pyrosynthase (ADPGPP) subunits, the granule bound and other starch synthase, the branching and debranching enzymes, the embryogenesis-abundant proteins, the gliadins, and the glutenins. Examples of such promoters in rice include those promoters for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the debranching enzymes, sucrose synthases, and the glutelins. A particularly preferred promoter is the promoter for rice glutelin, *Osgt-1* gene. Examples of such promoters for barley include those for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the debranching enzymes, sucrose synthases, the hordeins, the embryo globulins, and the aleurone specific proteins.

Root specific promoters may also be used. An example of such a promoter is the promoter for the acid chitinase gene (Samac *et al.*, Plant Mol. Biol. 25:587-596 (1994). Expression in root tissue could also be accomplished by utilizing the root specific subdomains of the CaMV 35S promoter that have been identified (Lam *et al.*, Proc. Natl. Acad. Sci. U.S.A. 86: 7890-7894 (1989). Other root cell specific promoters include those reported by Conkling *et al.* (Plant Physiol. 93: 1203-1211 (1990).

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous gene sequence of the DNA molecule of the present invention, and can be specifically modified if desired so as to increase translation of mRNA. For a review of optimizing expression of transgenes, see Koziel *et al.*, (Plant Mol. Biol. 32:393-405 (1996). The 5' non-translated regions can also be obtained from plant viral RNAs (Tobacco mosaic virus, Tobacco etch virus, Maize dwarf mosaic virus, Alfalfa mosaic virus, among others) from suitable eukaryotic genes, plant genes (wheat and maize chlorophyll a/b binding protein gene leader), or from a synthetic gene sequence. The

present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence. Leader sequences useful in context of the present invention comprise the maize Hsp70 leader

5 (U.S. Patent No. 5,362,865 and U.S. Patent No. 5,859,347), and the TMV omega element (Gallie *et al.*, The Plant Cell 1:301-311 (1989)).

A vector or construct may also include various regulatory elements. Intron sequences are known in the art to aid in the expression of transgenes in monocot plant cells. Examples of such introns include the *Adh* intron 1 (Callis *et al.*, Genes and Develop.

10 1:1183-1200 (1987), the sucrose synthase intron (Vasil *et al.*, Plant Physiol. 91:1575-1579 (1989), U.S. Patent No. 5,955,330), first intron of the rice actin gene (U.S. Patent No. 5,641,876).

A vector may also include a transit peptide nucleic acid sequence. The glyphosate target in plants, the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme, is

15 located in the chloroplast. Many chloroplast-localized proteins, including EPSPS, are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP) that is removed during the import steps. Examples of other such chloroplast proteins include the small subunit (SSU) of Ribulose-1,5,-bisphosphate carboxylase, Ferredoxin, Ferredoxin oxidoreductase, the light-harvesting

20 complex protein I and protein II, and Thioredoxin F. It has been demonstrated in vivo and in vitro that non-chloroplast proteins may be targeted to the chloroplast by use of protein fusions with a CTP and that a CTP sequence is sufficient to target a protein to the chloroplast. Incorporation of a suitable chloroplast transit peptide, such as, the

*Arabidopsis thaliana* EPSPS CTP (Klee *et al.*, Mol. Gen. Genet. 210:437-442 (1987), and the *Petunia hybrida* EPSPS CTP (della-Cioppa *et al.*, Proc. Natl. Acad. Sci. USA 83:6873-6877 (1986) has been shown to target heterologous EPSPS protein sequences to chloroplasts in transgenic plants. The production of glyphosate tolerant plants by

5 expression of a fusion protein comprising an amino-terminal CTP with a glyphosate resistant EPSPS enzyme is well known by those skilled in the art, (U.S. Patent No. 5,627,061, U.S. Patent No. 5,633,435, U.S. Patent No. 5,312,910, EP 0218571, EP 189707, EP 508909, and EP 924299). Those skilled in the art will recognize that various chimeric constructs can be made that utilize the functionality of a particular CTP to import

10 glyphosate resistant EPSPS enzymes into the plant cell chloroplast.

The termination of transcription is accomplished by a 3' non-translated DNA sequence operably linked in the chimeric vector to the gene of interest. The 3' non-translated region of a recombinant DNA molecule contains a polyadenylation signal that functions in plants to cause the addition of adenylate nucleotides to the 3' end of the

15 RNA. The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated region (Fraley *et al.*, Proc. Natl. Acad. Sci. 80:4803-4807 (1983), the 3' untranslated region from pea small subunit Rubisco gene (Coruzzi *et al.*, EMBO J. 3:1671-1679 (1994), the 3' untranslated region from soybean 7S seed storage protein gene (Schuler *et al.*, Nuc Acids Res. 10:8225-8244

20 (1982) are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

The aforesaid described genetic elements and other regulatory elements of similar function may be substituted when appropriate by those skilled in the art of plant molecular biology to provide necessary function to the plant expression cassette. DNA constructs for glyphosate tolerance designed for expression in plastids will necessarily contain genetic  
 5 elements that function in plastids.

A vector may also include a screenable or scorable marker gene. Screenable or scorable markers may be used to monitor expression. Exemplary markers include a  $\beta$ -glucuronidase or *uidA* gene (GUS) that encodes an enzyme for that various chromogenic substrates are known (Jefferson, Plant Mol. Biol, Rep. 5:387-405 (1987); Jefferson *et al.*,  
 10 EMBO J. 6:3901-3907 (1987); an R-locus gene, that encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, Stadler Symposium 11:263-282 (1988); a  $\beta$ -lactamase gene (Sutcliffe *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 75:3737-3741 (1978); a gene that encodes an enzyme for that various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a  
 15 luciferase gene (Ow *et al.*, Science 234:856-859 (1986); a xyle gene (Zukowsky *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 80:1101-1105 (1983) that encodes a catechol dioxygenase that can convert chromogenic catechols; an  $\alpha$ -amylase gene (Ikatu *et al.*, Bio/Technol. 8:241-242 (1990); a tyrosinase gene (Katz *et al.*, J. Gen. Microbiol. 129:2703-2714 (1983) that encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone  
 20 that in turn condenses to melanin; green fluorescence protein (Elliot *et al.*, Plant cell Rep. 18:707-714 (1999) and an  $\alpha$ -galactosidase.

Included within the terms "selectable or screenable marker genes" are also genes that encode a secretable marker whose secretion can be detected as a means of identifying

or selecting for transformed cells. Examples include markers that encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes that can be detected catalytically. Secretable proteins fall into a number of classes, including small, diffusible proteins that are detectable, (*e.g.*, by ELISA), small active enzymes that are

5 detectable in extracellular solution (*e.g.*,  $\alpha$ -amylase,  $\beta$ -lactamase, phosphinothricin transferase), or proteins that are inserted or trapped in the cell wall (such as proteins that include a leader sequence such as that found in the expression unit of extension or tobacco PR-S). Other possible selectable and/or screenable marker genes will be apparent to those of skill in the art.

10           There are many methods for introducing transforming nucleic acid molecules into plant cells. Suitable methods are believed to include virtually any method shown effective in introducing the nucleic acid molecules into a plant cell, such as by *Agrobacterium* infection or direct delivery of nucleic acid molecules.

Four general methods for direct delivery of a gene into cells have been described:

15 (1) chemical methods (Graham *et al.*, Virology 54:536-539 (1973); (2) physical methods such as microinjection (Capecchi, Cell 22:479-488 (1980); electroporation (Wong *et al.*, Biochem. Biophys. Res. Commun. 107:584-587 (1982); Fromm *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 82:5824-5828 (1985); (U.S. 5,384,253); and the gene gun (Johnston *et al.*, Methods Cell Biol. 43:353-365 (1994); (3) viral vectors (Clapp, Clin. Perinatol. 20:155-

20 168 (1993); Lu *et al.*, J. Exp. Med. 178:2089-2096 (1993); Eglitis *et al.*, Biotechniques 6:608-614 (1988); and (4) receptor-mediated mechanisms (Curiel *et al.*, Hum. Gen. Ther. 3:147-154 (1992), Wagner *et al.*, Proc. Natl. Acad. Sci. USA 89:6099-6103 (1992).

Acceleration methods that may be used include, for example, microprojectile bombardment and the like. One example of a method for delivering transforming nucleic acid molecules to plant cells is microprojectile bombardment. This method has been reviewed by Yang *et al.*, Particle Bombardment Technology for Gene Transfer, Oxford Press, Oxford, England (1994). Non-biological particles (microprojectiles) that may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly transforming monocots, is that neither the isolation of protoplasts (Cristou *et al.*, Plant Physiol. 87:671-674 (1988), nor the susceptibility of *Agrobacterium* infection are required. An illustrative embodiment of a method for delivering DNA into *Zea mays* cells by acceleration is a biolistics  $\alpha$ -particle delivery system, that can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension. Gordon-Kamm *et al.*, describes the basic procedure for coating tungsten particles with DNA (Gordon-Kamm *et al.*, Plant Cell 2:603-618 (1990). The screen disperses the tungsten nucleic acid particles so that they are not delivered to the recipient cells in large aggregates. A particle delivery system suitable for use with the present invention is the helium acceleration PDS-1000/He gun is available from Bio-Rad Laboratories (Bio-Rad, Hercules, California) (Sanford *et al.*, Technique 3:3-16 (1991).

For the bombardment, cells in suspension may be concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the

microprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded.

Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance  
5 below the microprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus that express the exogenous gene product 48 hours post-bombardment often range from one to ten and  
10 average one to three.

In bombardment transformation, one may optimize the pre-bombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the  
15 DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed  
20 that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

In another alternative embodiment, plastids can be stably transformed. Method disclosed for plastid transformation in higher plants include particle gun delivery of DNA



containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination (Svab *et al.* Proc. Natl. Acad. Sci. (U.S.A.) 87:8526-8530 (1990); Svab *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 90:913-917 (1993); (Staub *et al.*, EMBO J. 12:601-606 (1993). The methods disclosed in U.S. Patent No. 5, 451,513, U.S. Patent No. 5,545,818, U.S. Patent No. 5,877,402, U.S. Patent No. 5,932,479, and WO 99/05265.

Accordingly, it is contemplated that one may wish to adjust various aspects of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors by modifying conditions that influence the physiological state of the recipient cells and that may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

*Agrobacterium*-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example the methods described by Fraley *et al.*, Bio/Technology 3:629-635 (1985) and Rogers *et al.*, Methods Enzymol. 153:253-277 (1987). Further, the integration of the T-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border

sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, Mol. Gen. Genet. 205:34 (1986).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*,  
5 In: Plant DNA Infectious Agents, Hohn and Schell, eds., Springer-Verlag, New York, pp. 179-203 (1985). Moreover, technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described have convenient multi-linker regions flanked by a  
10 promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes (Rogers *et al.*, Methods Enzymol. 153:253-277 (1987). In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant varieties where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined  
15 nature of the gene transfer.

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single genetic locus on one chromosome. Such transgenic plants can be referred to as being hemizygous for the added gene. More preferred is a transgenic plant that is homozygous for the added structural gene; *i.e.*, a transgenic plant that contains two  
20 added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants for the gene of interest.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in Fehr, In: *Breeding Methods for Cultivar Development*, Wilcox J. ed., American Society of Agronomy, Madison WI (1987).

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, e.g., Potrykus *et al.*, Mol. Gen. Genet. 205:193-200 (1986); Lorz *et al.*, Mol. Gen. Genet. 199:178 (1985); Fromm *et al.*, Nature 319:791 (1986); Uchimiya *et al.*, Mol. Gen. Genet. 204:204 (1986); Marcotte *et al.*, Nature 335:454-457 (1988). Application of these systems to different plant varieties depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura *et al.*, Plant Tissue Culture Letters 2:74 (1985); Toriyama *et al.*, Theor Appl. Genet. 205:34 (1986); Yamada *et al.*, Plant Cell Rep. 4:85 (1986); Abdullah *et al.*, Biotechnology 4:1087 (1986).

Other methods of cell transformation can also be used and include but are not limited to introduction of DNA into plants by direct DNA transfer into pollen (Hess *et al.*, Intern Rev. Cytol. 107:367 (1987); Luo *et al.*, Plant Mol Biol. Reporter 6:165 (1988), by direct injection of DNA into reproductive organs of a plant (Pena *et al.*, Nature 325:274

(1987), or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos (Neuhaus *et al.*, Theor. Appl. Genet. 75:30 (1987).

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art  
5 (Weissbach *et al.*, In: *Methods for Plant Molecular Biology*, Academic Press, San Diego, CA, (1988). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in  
10 an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene is well known in the art. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from  
15 plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired exogenous nucleic acid is cultivated using methods well known to one skilled in the art.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have been published for cotton (US Patent No. 5,004,863,  
20 US Patent No. 5,159,135, US Patent No. 5,518,908); soybean (US Patent No. 5,569,834, US Patent No. 5,416,011, McCabe *et. al.*, Bio/Technology 6:923 (1988), Christou *et al.*, Plant Physiol. 87:671-674 (1988); Brassica (U.S. 5,463,174); peanut (Cheng *et al.*, Plant

Cell Rep. 15:653-657 (1996), McKently *et al.*, Plant Cell Rep. 14:699-703 (1995); and pea (Grant *et al.*, Plant Cell Rep. 15:254-258, (1995).

Transformation of monocotyledons using electroporation, particle bombardment, and *Agrobacterium* have also been reported. Transformation and plant regeneration have  
 5 been achieved in asparagus (Bytebier *et al.*, Proc. Natl. Acad. Sci. (USA) 84:5354-5349 (1987); barley (Wan *et al.*, Plant Physiol 104:37-48 (1994); Zea mays (Rhodes *et al.*, Science 240:204-207 (1988), Gordon-Kamm *et al.*, Plant Cell 2:603-618 (1990), Fromm *et al.*, Bio/Technology 8:833-839 (1990), Koziel *et al.*, Bio/Technology 11:194-200 (1993), Armstrong *et al.*, Crop Science 35:550-557 (1995); oat (Somers *et al.*,  
 10 Bio/Technology 10:1589-1594 (1992); orchard grass (Horn *et al.*, Plant Cell Rep. 7:469-472 (1988); rice (Toriyama *et al.*, Theor Appl. Genet. 205:34- (1986), Part *et al.*, Plant Mol. Biol. 32:1135-1148, (1996), Abedinia *et al.*, Aust. J. Plant Physiol. 24:133-141 (1997), Batraw *et al.*, Plant Mol. Biol. 15:527-538 (1990), Christou *et al.*, Bio/Technology 9:957-962 (1991); rye (De la Pena *et al.*, Nature 325:274-276 (1987);  
 15 sugarcane (Bower *et al.*, Plant J. 2:409-416 (1992); tall fescue (Wang *et al.*, Bio/Technology 10:691-696 (1992); and wheat (Vasil *et al.*, Bio/Technology 10:667-674 (1992); US Patent No. 5,631,152).

Assays for gene expression based on the transient expression of cloned nucleic acid vectors have been developed by introducing the nucleic acid molecules into plant cells by  
 20 polyethylene glycol treatment, electroporation, or particle bombardment (Marcotte *et al.*, Nature 335:454-457 (1988); Marcotte *et al.*, Plant Cell 1:523-532 (1989); McCarty *et al.*, Cell 66:895-905 (1991); Hattori *et al.*, Genes Dev. 6:609-618 (1992); Goff *et al.*, EMBO J. 9:2517-2522 (1990). Transient expression systems may be used to functionally dissect

gene constructs (see generally, Mailga *et al.*, Methods in Plant Molecular Biology, Cold Spring Harbor Press (1995). It is understood that any of the nucleic acid molecules of the present invention can be introduced into a plant cell in a permanent or transient manner in combination with other genetic elements such as promoters, leaders, transit peptide  
 5 sequences, enhancers, introns, 3' nontranslated regions and other elements known to those skilled in the art that are useful for control of transgene expression in plants.

*Eleusine indica* has been shown to hybridize with *Eleusine coracana* (finger millet), an important cultivated millet of India and East Africa (Chennaveeraiah et al., Euphytica 2-3:489-495, (1974). Classical plant breeding methods can be used to transfer  
 10 the gene and the glyphosate tolerant phenotype to crop plants within the family Poaceae. The DNA molecules of the EPSPS glyphosate resistance gene of *E. indica* (SEQ ID NO:6) can be used as a probe to identify other like DNA molecules by standard methods. Oligonucleotide DNA molecules homologous or complementary to the EPSPS glyphosate resistance gene of *E. indica* can be used in a marker assisted breeding method (Simple  
 15 sequence repeat DNA marker analysis, in "DNA markers: Protocols, applications, and overviews: (1997) 173-185, Cregan, et al., eds., Wiley-Liss NY ) to assist in the breeding of this gene into related and heterologous crop species.

In addition to the above discussed procedures, practitioners are familiar with the standard resource materials that describe specific conditions and procedures for the  
 20 construction, manipulation and isolation of macromolecules (*e.g.*, DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolating of clones, (see for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989); Mailga *et al.*, Methods in Plant Molecular Biology, Cold

Spring Harbor Press (1995); Birren *et al.*, Genome Analysis: Detecting Genes, 1, Cold Spring Harbor, New York (1998); Birren *et al.*, Genome Analysis: Analyzing DNA, 2, Cold Spring Harbor, New York (1998); Clark *et al.*, Plant Molecular Biology: A Laboratory Manual, Springer, New York (1997); and Innis *et al.*, PCR Protocols: A  
 5 Guide to Methods and Applications, Academic Press: San Diego, (1990).

Plant species containing a naturally occurring EPSPS enzyme resistant to glyphosate have not been previously reported. The subject of this invention is the EPSPS enzyme isolated from *Eleusine indica* that has been shown to be resistant to glyphosate and the expression of the DNA molecule encoding this EPSPS enzyme in other plants that  
 10 then confers glyphosate tolerance to those recipient plants. The glyphosate resistant EPSPS enzyme isolated from *Eleusine indica* glyphosate tolerant biotype has a novel  $K_m$  with respect to binding of PEP as compared to other plant EPSP Synthases that have been modified for glyphosate resistance by a single amino acid substitution of a proline to serine substitution in the active site of the enzyme. The  $K_m$  for PEP of the *E. indica* glyphosate  
 15 resistant enzyme is little changed from the *E. indica* glyphosate sensitive EPSPS enzyme. In addition, this gene is from a monocot plant and hence may not need nucleic sequence modification to affect expression in transgenic monocot crop plants. The *E. indica* glyphosate tolerant EPSPS enzyme amino acid sequence can be modified by site directed mutation to include other known substitutions.

20 The present invention also provides for parts of the plants of the present invention. Plant parts, without limitation, include seed, endosperm, ovule and pollen. In a particularly preferred embodiment of the present invention, the plant part is a seed.

The following examples are included to demonstrate examples of certain preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to

5 constitute examples of preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. All references cited herein are hereby expressly incorporated herein by reference.

10 Seeds from glyphosate tolerant *Eleusine indica* plants were deposited with the American Type Culture Collection (ATCC, 10801 University Blvd, Manassas, Virginia, U.S.A., 20110-2209) and assigned ATCC No. PTA-2177. The deposit will be maintained in the depository for a period of 30 years, or 5 years after the last request, or for the effective life of the patent, whichever is longer, and will be replaced as necessary during

15 that period.

#### EXAMPLE 1

*Eleusine indica* plants tolerant to glyphosate were collected from a site near Johor, Malaysia. Glyphosate tolerant biotypes of *E. indica* are identified and numbered. Seed is collected from each biotype and planted in pots in the greenhouse. Clones are generated

20 for each plant by excising 10-20 tillers and transplanting these in separate pots.

Glyphosate sensitive and tolerant individual plants are then identified by treatment with glyphosate at either 0.5 kg active ingredient (ai)/hectare (ha) or 2.0 kg ai/ha, respectively. A corresponding clone for glyphosate tolerant and glyphosate sensitive *E. indica* biotype



is left untreated. These clones are used as the source of fresh tissue for enzyme analysis and gene isolation.

Construction of a cDNA library from the glyphosate tolerant *E. indica* biotype and the glyphosate sensitive *E. indica* biotype is performed by isolating total RNA from the crown tissues. The crown tissues are dissected from the plants, then flash-frozen with liquid nitrogen and maintained at -80°C until needed. Total RNAs are extracted from frozen crown samples using the RNeasy Plant Mini Kit (cat.# 74904, Qiagen Inc., Valencia, CA) per manufacturer's instructions. Oligo'dT-primed first-strand cDNAs are prepared from 5 µg samples of total RNA using the Superscript Pre-Amplification System (cat.# 18089-011, Life Technologies, Rockville, MD) per manufacturer's instructions. Two µl of first-strand cDNA are then used to generate partial *E. indica* EPSP synthase cDNAs via polymerase chain reaction using a modification of the "touchdown PCR" technique (Don *et al.*, Nucl. Acids Res. 19:4008, 1991). Degenerate oligonucleotide pools of SEQ ID NO:1 and SEQ ID NO:2 are added in a 50 µl RT-PCR reaction at a final concentration of 25 µM.

5'-TNWSNGTNGARGCNGAYAARGT-3' (SEQ ID NO: 1)

5'-GCCATNGCCATNCKRTGRTCRTC-3' (SEQ ID NO: 2)

PCR amplifications are then performed using the Expand High Fidelity PCR System (cat.# 1 732 641, Roche Molecular Biochemicals, Indianapolis, IN) per manufacturer's instructions. A thermal profile of 94°C for 20 seconds, followed by 60°C for 1 minute, then 72°C for 1 minute 30 seconds is used for the initial 30 cycles with a 0.5°C decrease in annealing temperature per cycle. This is followed by 10 additional cycles of 94°C for 20 seconds, 45°C for 1 minute, then 72°C for 1 minute 30 seconds.

RT-PCR products are then purified by agarose gel electrophoresis using a QIAquick Gel Extraction Kit (cat.# 28704, Qiagen Inc., Valencia, CA) then directly cloned into the pCR2.1-TOPO vector (cat.# K4500-40, Invitrogen, Carlsbad, CA). The identity of the cloned RT-PCR products is confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster City, CA ).

The remainder of the 3' end of the EPSP synthase coding region is generated using the 3' RACE System for Rapid Amplification of cDNA Ends (cat.# 18373-027, Life Technologies, Rockville, MD), using the gene-specific oligonucleotide of SEQ ID NO:3. The cDNA is prepared according to manufacturer's instructions using 5 µg of total RNA isolated from crown tissues as previously described.

5'-GTGAAAGCAGAGCATTCTGATAGC-3' (SEQ ID NO: 3)

PCR amplifications are conducted in 50 µl reactions including 5 µl first-strand cDNA reaction, 20 picomoles of each primer, 10 mM TrisHCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, and 2.5 units Taq polymerase. A thermal profile of 94°C for 20 seconds, followed by 57°C for 1 minute, then 72°C for 1 minute 30 seconds is used for 35 cycles. The identity of the 3'-RACE products is confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster, CA ).

The remainder of the 5' end of the *E. indica* EPSP synthase mature protein coding region is generated using the SMART RACE cDNA Amplification Kit (cat.# K1811-1, Clontech Laboratories Inc., Palo Alto, CA), using the gene-specific oligonucleotides of SEQ ID NO: 4 and SEQ ID NO:5. The cDNA is prepared according to manufacturer's instructions using 150 ng of polyA<sup>+</sup> mRNA isolated from crown tissues using an Oligotex mRNA Midi Kit (cat.# 28704, Qiagen Inc., Valencia, CA).

5'-GGCTGCTGTCAATGTCGCATTGCAGTTCC-3' (SEQ ID NO: 4)

5'-CTCTTTCGCATCCTTCTCAACTGGGAACTTGC-3' (SEQ ID NO: 5)

PCR reactions are conducted as recommended by the manufacturer, except that the Expand High Fidelity PCR System (cat.# 1 732 641, Roche Molecular Biochemicals, Indianapolis, IN) is used and DMSO is included in all reactions at a final concentration of 5.0% to facilitate the amplification of GC-rich sequences. The synthetic DNA oligonucleotide described in SEQ ID NO: 4 is used in the primary amplifications, then second round ("nested") amplifications are performed using the oligonucleotide described in SEQ ID NO: 5, with a 1 µl aliquot of 1:100 dilution of the primary PCR reactions. The identity of the 5'-RACE products is confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster City, CA ).

The significant overlap of sequences generated by RT-PCR, 3' RACE, and 5' RACE allows for the unambiguous assembly of the sequences into a single DNA sequence containing the entire open reading frame for the mature protein, using the SEQMan II software package (DNASTAR Inc., Madison, WI). The DNA sequence corresponding to the mature protein-coding region of the *Eleusine indica* (glyphosate tolerant biotype) EPSP synthase gene (SEQ ID NO:6) is shown in Figure 1.

The deduced amino acid sequence for the mature protein-coding region of the *Eleusine indica* (glyphosate tolerant biotype) EPSP synthase gene (SEQ ID NO:7) for this protein is shown in Figure 2.

## EXAMPLE 2

EPSP synthase enzyme from the glyphosate tolerant *E. indica* biotype confers increased glyphosate tolerance in transgenic *E. coli*. *E. coli* is useful as a heterologous

expression system for testing glyphosate resistant enzymes. The EPSP synthase mature protein-coding regions isolated from the glyphosate tolerant and glyphosate sensitive *E. indica* biotypes, can be directly compared for their ability to confer tolerance to glyphosate in transgenic hosts. *E. coli* (strain SR481) are transformed with the

5 glyphosate resistant EPSPS gene (Ei.EPSPS:glyR) and the glyphosate sensitive EPSPS gene (Ei.EPSPS:glyS) purified from *E. indica*. The growth rate differentials of transformed cell lines grown in the presence of glyphosate contained in the culture medium is used as a measure of the resistance of the EPSPS enzyme to the inhibitory effects of glyphosate (Rogers *et al.*, Appl. Environ. Microbiol. 46:37-43 (1983). An

10 appropriate *E. coli* expression vector that carries the native promoter and operator sequence from the *E. coli* lac operon (Dickson *et al.*, Science 187:27-35 (1975) including the sequence

5'-AGATCTCCTAGGGCTTAATTAATTAAGCACTAGTCACACAGG  
AGGTAATTCATATG- 3' (SEQ ID NO:8)

15 is contained in pMON45337. This nucleotide sequence includes 1) flanking BglII and NdeI endonuclease sites, 2) a ribosome binding site, and 3) an unstructured region 5' to the ribosome binding element (Balbas, P. et.al., in "Methods in Enzymology" (D. V. Goeddel, ed.)185: 15-37, 1990). This was inserted by ligation to facilitate expression and cloning at the ATG start codon of an open reading frame. A multiple cloning site is

20 positioned immediately downstream of this NdeI site, followed by the rho-independent transcriptional terminator element of the *E. coli trpA* gene (T. Sato *et al.*, J. Biochem. (Tokyo), 101:525-534, (1987). This vector when it operably contains the EPSP synthase coding sequences of the present invention is employed for the inducible expression of

glyphosate resistant and glyphosate sensitive EPSP synthase cDNAs in *E. coli*. Other commercially available inducible *E. coli* expression vectors are suitable for testing the EPSP synthases from *E. indica*.

DNA manipulations and transformations of *E. coli* are performed according to standard procedures (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989). To construct *E. coli* expression vectors carrying the EPSP synthase mature protein coding sequences from the tolerant and sensitive *E. indica* biotypes, the oligonucleotide primers of SEQ ID NO:9 and SEQ ID NO:10.

5'-GCAATTCCATATGGCGGGCGCGGAGGAGGTGGTGCT - 3' (SEQ ID NO: 9)

10 5'-GACTAGGAATTCTTAGTTCTTTTGACGAAAGTGCTCAGCACGTCGAAG-3'  
(SEQ ID NO: 10),

These sequences are employed in RT-PCR reactions to generate expression cassettes suitable for cloning into pMON45337 cut with the restriction enzymes NdeI and EcoRI. RT-PCR reactions are performed with total RNAs extracted from frozen crown samples using the RNeasy Plant Mini Kit (cat.# 74904, Qiagen Inc., Valencia, CA) per manufacturer's instructions. Oligo dT-primed first-strand cDNAs are prepared from 5 µg samples of total RNA using the Superscript Pre-Amplification System (cat.# 18089-011, Life Technologies, Rockville, MD) per manufacturer's instructions. Two µl of first-strand cDNA are then used to generate *E. indica* EPSP synthase expression cassettes via polymerase chain reaction. The oligonucleotides are added in 50 µl RT-PCR reactions at a final concentration of 0.4 µM. PCR amplifications are then performed using the Expand High Fidelity PCR System (cat.# 1 732 641, Roche Molecular Biochemicals, Indianapolis, IN) per manufacturer's instructions, using a thermal profile of 94°C for 30 seconds, then

57°C for 2 minutes, followed by 75°C for 3 minutes, for a total of 35 cycles. The resulting PCR products are digested with Nde I and EcoRI, then ligated into pMON45337, resulting in the *E. coli* expression vectors pMON45364 (Figure 5) and pMON45365 (Figure 6), which contain the mature protein coding region for *E. indica* EPSP synthase isolated from the resistant and sensitive biotype, respectively. Expression of the two enzymes in *E. coli* will thus be directed by the Lac operon and trpA gene genetic elements described above for pMON45337. The accuracy of the cloned sequences are confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster, CA ). pMON45337, pMON45364, and pMON45365 are all transformed into the *E. coli* strain SR481, an *aroA*- strain lacking endogenous EPSP synthase activity (Padgett *et al.*, Arch. Biochem. Biophys. 258:564-573 (1987).

To directly compare the glyphosate tolerance of *E. coli aroA*- cells expressing the EPSP synthase gene isolated from the glyphosate sensitive *E. indica* biotype with cells expressing the EPSP synthase gene isolated from the glyphosate tolerant biotype, growth rates are compared for the two cell lines in the presence of increasing concentrations of glyphosate (Figure 3). Growth rates are also monitored for *E. coli* SR481 cells transformed with pMON45337 (empty vector) in the absence of glyphosate as a negative control. Fresh overnight cultures of *E. coli* SR481 cells transformed with pMON45337, pMON45364 (Ei.EPSPS:glypR), and pMON45365 (Ei.EPSPS:glypS) are grown in Terrific Broth (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989), supplemented with 1.0 mM IPTG, 50 µg/ml ampicillin, and 100 µg/ml each of L-phenylalanine, L-tyrosine, and L-tryptophan. O.D.<sub>595</sub> measurements are taken on all of the overnight cultures to confirm similar cell densities. For *E. coli* SR481-

pMON45364 and SR481-pMON45365 cells, 14 ml culture tubes (cat. # 60818-725, VWR Scientific, West Chester, PA) each containing 3.0 ml of minimal M9 media (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989) supplemented with 50 µg/ml ampicillin, 1.0 mM IPTG, and either 0.0, 0.5, 1.5, or 5.0 mM  
 5 glyphosate (N-phosphonomethyl glycine or a salt thereof) are inoculated with 100 µl of undiluted overnight culture per tube. Each experimental condition is performed in triplicate to confirm the reproducibility of the experiment. Where  $t = 0, 12, 24, 28, 32,$  and 48 hours past the onset of growth in minimal media, 100 µl aliquots from each tube are removed and O.D.<sub>595</sub> measurements are taken immediately. A typical result from these  
 10 analyses is shown in Figure 3, where an approximately three-fold increase in tolerance to glyphosate is observed due to the expression of the EPSP synthase enzyme from the glyphosate tolerant *E. indica* biotype.

### EXAMPLE 3

Kinetic characterization of the *E. indica* glyphosate-resistant EPSP synthase  
 15 activity in plant and bacterial extracts. Kinetic characterization of the glyphosate-resistant *E. indica* enzyme are performed using both partially purified plant extracts as well as bacterial extracts prepared from cells expressing the cloned sequence on a suitable vector such as pMON45365. Parameters that describe the enzyme's resistance to glyphosate-mediated inhibition and the affinity for the substrate phosphoenolpyruvate (PEP) are of  
 20 particular interest, given that glyphosate is a competitive inhibitor of EPSP synthase with respect to PEP (Boocock, M. *et al.*, FEBS Letts. 154:127-133 (1983).

Preparation of extracts and radiometric EPSP synthase assays are performed using methods adapted from published procedures (Padgett *et al.*, J. Biol. Chem. 266:22364-

22369 (1991). Crown regions are dissected from whole plants, pulverized under liquid nitrogen with a mortar and pestle, then stored at -80°C prior to extraction. Homogenates are prepared from 0.5 g tissue per sample in 25 ml extraction buffer (100 mM TrisCl, 10 % glycerol, 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride HCl, 0.1 mM leupeptin, pH 7.4) at 4°C using a model PT3000 Polytron homogenizer (Brinkman Instruments Inc., Westbury, NY). Debris is removed by 0.2 µm filtration, then the resulting supernatant is concentrated and desalted using an Ultrafree-15 centrifugal filtration unit (cat.# UFV2-BGC-10, Millipore Corp., Bedford, MA). Final sample volumes are approximately 0.5 ml. Protein concentrations are determined spectrophotometrically using the Bio-Rad protein assay reagent (cat.#500-0006, Bio-Rad Laboratories, Hercules, CA). EPSPS specific activities are determined by assaying 10 µl extract at 25°C for 5- 15 min. (50 µl reactions include 50 mM HEPES, pH 7.0, 5 mM potassium fluoride, 1 mM shikimate-3-phosphate, 0.5 mM [1-<sup>14</sup>C]-phosphoenolpyruvate (29.0 mCi/mmol cyclohexylammonium salt; #CFQ10004, Amersham Life Science, Inc., Arlington Heights, IL), and 0.1 mM ammonium molybdate). Reactions are quenched with the addition of 50 µl 9:1 ethanol: 0.1 M acetic acid. Thirty µl of quenched reaction is then injected onto a Synchropak AX100 anion exchange column (cat.# 942804, P.J. Cobert Associates, Inc., St. Louis, MO) equilibrated with 0.235 M potassium phosphate buffer, pH 6.5, and eluted isocratically with the same buffer. A model D525 radioactive flow detector (Packard Instrument Co., Downer's Grove, IL) is used to determine production of [<sup>14</sup>C]-EPSP in the reaction.

For determination of I<sub>50</sub> (glyphosate) values, the assays as described, are performed in the presence of increasing concentrations of glyphosate and the resulting activities



analyzed and plotted using GraFit version 3.0 software (Erithacus Software Ltd., Staines, U.K.). Figure 4 shows data generated for a typical glyphosate inhibition study, comparing the EPSP synthase activities detectable in extracts prepared from the glyphosate sensitive and tolerant *E. indica* biotypes. These data demonstrate the difference in sensitivity to glyphosate for the activities present in the two biotypes, with the sensitive biotype EPSPS activity having an  $I_{50}$  (glyphosate) of approximately 3.0  $\mu\text{M}$  and the tolerant biotype EPSPS approximately 16  $\mu\text{M}$ .

Activities of the enzymes extracted from several different tolerant and sensitive *E. indica* individuals are compared in the presence and absence of 1.6  $\mu\text{M}$  glyphosate (Table 1), showing similar sensitivity to glyphosate and very low plant-to-plant variation exhibited among individuals from the same biotype.

Table 1. Percent-maximal EPSPS activity in extracts from different *E. indica* individuals assayed in the presence 1.6  $\mu\text{M}$  glyphosate

biotype-individual	EPSPS Activity, (% maximal @ 1.6 $\mu\text{M}$ glyphosate)
Sensitive- #1	51.6
Sensitive- #2	55.6
Sensitive- #3	57.4
Tolerant - #1	76.3
Tolerant - #2	86.0
Tolerant - #3	82.4

Similar analyses are performed using extracts prepared from *E. coli* strain SR481 cells expressing the cloned *E. indica* sensitive and resistant EPSPS enzymes from the expression vectors pMON45365 and pMON45364, respectively. Fresh bacterial overnight cultures are grown at 37°C in Terrific Broth, (Sambrook *et al.*, Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989), supplemented with 50  $\mu\text{g/ml}$  ampicillin and 100  $\mu\text{g/ml}$  each of L-phenylalanine, L-tyrosine, and L-tryptophan.

Overnight cultures are used to inoculate large-scale cultures containing the same media at a 1:100 dilution, then grown to an O.D.<sub>595</sub> of 0.6 at 37°C with vigorous shaking. The

5 culture is induced by the addition of IPTG to 1.0 mM final concentration then incubation is continued for an additional 4 hours. Cells are then pelleted by centrifugation at 10,000 x g for 5 minutes, then washed twice in ice-cold 0.9% NaCl. Excess wash buffer is

removed by aspiration, then pellets are flash-frozen in liquid nitrogen and stored at -80°C prior to use. Bacterial extracts are prepared using the same extraction buffer used for

10 plant extracts, with 3 ml buffer added per gram of pelleted cells. Cells are lysed using a French press (model # J5-598A, American Instrument Co., Silver Springs, MD.), then extracts are centrifuged at 14,000 x g for 10 minutes to remove cell debris. The

supernatants are then desalted using a PD-10 column (cat.# 17-0851-01, Amersham Pharmacia Biotech Inc., Piscataway, NJ). EPSP synthase assays are performed as

15 described above for plant-derived extracts. Table 2 illustrates a typical result for bacterial extracts expressing the sensitive and resistant *E. indica* EPSP synthase enzymes assayed in the presence and absence of 1.6  $\mu\text{M}$  glyphosate. These data indicate that similar inhibition kinetics are obtained when these results are compared to the respective activities detected in plant extracts.

Table 2. EPSPS activity in *aroA*- bacterial extracts transformed with pMON45364 or pMON45365 assayed in the presence and absence of glyphosate

sample	activity (nMol/min/mg protein)	% maximal activity (1.6 uM glyphosate)
SR481(pMON45364)		
- glyphosate	34.3	
+ glyphosate	24.9	72.6
SR481(pMON45365)		
- glyphosate	27.1	
+ glyphosate	14.4	53.1

#### EXAMPLE 4

The use of the glyphosate resistant EPSP synthase gene from *E. indica*

(*Ei*.EPSPS:glypR) to develop glyphosate tolerant crop plants involves the construction of

5 plant transformation vectors that include the appropriate combination of genetic elements

necessary to direct adequate expression levels in target tissues. For monocotyledonous

crop plants, a monocot vector that utilizes a plant expression cassette that contains a

promoter (P) and first intron (I) from the rice (*Os*) actin gene (P-*Os*.Act1/I-*Os*.Act1 (U.S.

Patent No. 5,641,876), plastid transit peptide sequence (TS) from the *Arabidopsis*

10 *thaliana* (*At*) EPSP synthase gene (TS-*At*.EPSPS:CTP) (Klee *et al.* Mol. Gen. Genet.

210:437-442), a *E. indica* glyphosate resistant EPSPS coding sequence, and the

polyadenylation/termination (T) region from the *Agrobacterium tumefaciens* nopaline

synthase gene (T-AGRTU.nos), would be an appropriate choice. This expression cassette

may be combined with a second transgene expression cassette by plant breeding, plant

15 transformation, or by joining in a DNA construct that comprises a plant DNA virus

promoter, for example, the cauliflower mosaic virus (CaMV) 35S promoter containing a

tandem duplication of the enhancer region, operably connected to a *Zea mays* Hsp70 intron, operably connected to a nucleic acid sequence encoding an *Arabidopsis thaliana* EPSPS chloroplast transit peptide sequence, operably connected to a *E. indica* glyphosate resistant EPSPS coding sequence, operably connected to a nopaline synthase

5 transcriptional terminator. Other combinations of genetic elements are known and those skilled in the art of plant molecular biology can easily construct plant expression vectors that will express the *E. indica* EPSPS glyphosate resistant enzyme at sufficient levels to confer glyphosate the transformed plant.

For specific dicotyledonous species, a plant expression vector that utilizes the  
 10 promoter and 5' untranslated region (including intron I) of the plant elongation factor 1 $\alpha$  gene (Elf $\alpha$ -A1) as described in U.S. Patent No. 5,177,011 or more specifically, dicot vector of the present invention which utilizes the *Arabidopsis thaliana* Elf $\alpha$ -A1 promoter and intron sequence (P-At.Elf1a/I-At.Elf1a), (Axelos *et al.*, Mol. Gen. Genet. 219: 106-112 (1989); Genbank accession # U63815), the chloroplast transit peptide from the  
 15 *Arabidopsis thaliana* EPSP synthase gene (TS-At.EPSPS:CTP2), and the polyadenylation/3' termination region from the *Pisum sativum* ribulose-1,5-bisphosphate carboxylase gene (T-Ps.RbcS:E9). This expression cassette may be combined with a second transgene expression cassette by plant breeding, plant transformation, or by joining in a DNA construct that comprises a plant DNA virus promoter, for example, the Figwort  
 20 mosaic virus (FMV) 34S promoter, operably connected to a nucleic acid sequence encoding an *Arabidopsis thaliana* EPSPS chloroplast transit peptide sequence, operably connected to a *E. indica* glyphosate resistant EPSPS coding sequence, operably connected to a nopaline synthase transcriptional terminator. Other combinations of genetic elements

are known and those skilled in the art of plant molecular biology can easily construct plant expression vectors that will express the *E. indica* EPSPS glyphosate resistant enzyme at sufficient levels to confer glyphosate tolerance to the transformed plant.

To construct monocotyledonous and dicotyledonous plant expression vectors  
 5 carrying the DNA encoding the EPSP synthase mature protein coding sequence isolated from the glyphosate tolerant *E. indica* biotype (SEQ ID NO. 6, Figure 1), the oligonucleotide primers of SEQ ID NO: 11 and SEQ ID NO: 12 are employed in PCR reactions to generate an expression cassette suitable for direct cloning into a monocot vector and a dicot vector.

10 5'-GCAATTCGCATGCCGGGCGCGGAGGAGGTGGTGCT-3' (SEQ ID NO: 11)  
 5'-GACTAGGAATTCTTAGTTCTTTTGACGAAAGTGCTCAGCACGTCGAAG - 3'  
 (SEQ ID NO: 12)

The PCR reactions are performed using 300-500 ng of pMON45364 plasmid DNA as template to amplify the *E. indica* EPSP synthase mature protein coding region,  
 15 flanked by SphI and EcoRI restriction cleavage sites. The oligonucleotides are added in 50 µl PCR reactions at a final concentration of 0.4 µM. PCR amplifications are then performed using the Expand High Fidelity PCR System (cat.# 1 732 641, Roche Molecular Biochemicals, Indianapolis, IN) per manufacturer's instructions, using a thermal profile of 94°C for 30 seconds, then 57°C for 2 minutes, followed by 75°C for 3 minutes,  
 20 for a total of 20-35 cycles. The resulting PCR products are digested with SphI and EcoRI, then ligated into pMON45366 and pMON45368 resulting in the plant expression vectors pMON45367 (Figure 7) and pMON45369 (Figure 8), respectively. The accuracy

of the cloned sequences are confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster City, CA).

### EXAMPLE 5

Transgenic corn can be produced by particle bombardment transformation methods as described in U.S. Patent No. 5,424,412. The plant expression vector (pMON45367) contains the glyphosate resistant *E. indica* EPSPS mature protein coding sequence in an expression cassette suitable for expression in monocot plants. The pMON45367 plasmid DNA is digested with NotI and PmeI restriction endonucleases to complete digestion. The 3.3 kb expression cassette is agarose gel purified, then bombarded into embryogenic corn tissue culture cells using a Biolistic® (Dupont, Wilmington, DE) particle gun with purified isolated DNA fragment. Transformed cells are selected on glyphosate (N-phosphonomethyl glycine and its salts) containing media and whole plants are regenerated then grown under greenhouse conditions. Fertile seed is collected, planted and the glyphosate tolerant phenotype is back crossed into commercially acceptable corn germplasm by methods known in the art of corn breeding (Sprague *et al.*, Corn and Corn Improvement 3<sup>rd</sup> Edition, Am. Soc. Agron. Publ (1988).

Transgenic corn plants can be produced by an *Agrobacterium* mediated transformation method. A disarmed *Agrobacterium* strain C58 (ABI) harboring a binary vector (pMON45367) is used for all the experiments. The pMON45367 is transferred into *Agrobacterium* by a triparental mating method (Ditta *et al.*, Proc. Natl. Acad. Sci. 77:7347-7351). Liquid cultures of *Agrobacterium* are initiated from glycerol stocks or from a freshly streaked plate and grown overnight at 26°C-28°C with shaking (approximately 150 rpm) to mid-log growth phase in liquid LB medium, pH 7.0 containing

50 mg/l kanamycin, 50 mg/l streptomycin and spectinomycin and 25 mg/l chloramphenicol with 200  $\mu$ M acetosyringone (AS). The *Agrobacterium* cells are resuspended in the inoculation medium (liquid CM4C) and the density is adjusted to OD<sub>660</sub> of 1. Freshly isolated Type II immature HiIIxLH198 and HiII corn embryos are inoculated with

5 *Agrobacterium* containing pMON45367 and co-cultured 2-3 days in the dark at 23 °C.

The embryos are then transferred to delay media (N6 1-100-12/micro/Carb 500/20  $\mu$ M AgNO<sub>3</sub>) and incubated at 28 °C for 4 to 5 days. All subsequent cultures are kept at this

temperature. Coleoptiles are removed one week after inoculation. The embryos are

transferred to the first selection medium (N61-0-12/Carb 500/0.5 mM glyphosate). Two

10 weeks later, surviving tissue are transferred to the second selection medium (N61-0-

12/Carb 500/1.0 mM glyphosate). Subculture surviving callus every 2 weeks until events

can be identified. This will take 3 subcultures on 1.0 mM glyphosate. Once events are

identified, bulk up the tissue to regenerate. For regeneration, callus tissues are transferred

to the regeneration medium (MSOD 0.1  $\mu$ M ABA) and incubated for two weeks. The

15 regenerating calli are transferred to a high sucrose medium and incubated for two weeks.

The plantlets are transferred to MSOD media in culture vessel and kept for two weeks.

Then the plants with roots are transferred into soil.

Three R<sub>0</sub> plants are regenerated for any given transgenic event. These three plants are expected to be near isogenic because they are thought to be derived from a single

20 transgenic plant cell. Thus, one plant is used as a non-sprayed control and the remaining

two plants are treated with glyphosate (as Roundup® herbicide). Plants are most

effectively treated with glyphosate at V2-V6 stage. Glyphosate (as Roundup® herbicide)

is administered through the use of a linear track sprayer set to deliver a 16, 32 or 64 oz./A

rate of glyphosate. Vegetative tolerance to the glyphosate is visually evaluated a week after spray based on a scale of 0 to 5 (0 = No observable/vegetative effect of glyphosate; 1 = Chlorosis observed; 2 = Advanced chlorosis, minor necrosis; 3 = Advanced chlorosis, moderate necrosis; 4 = Advanced chlorosis, severe necrosis; 5 = No live tissue remaining).

- 5 The  $R_0$  plants produced are allowed to self, then  $R_1$  plants are screened using spray applications of glyphosate and the rating system as described for the  $R_0$  screen. An increase in whole-plant tolerance to the herbicide, as compared to non-transgenic control plants, is used to assess the utility of the *E. indica* EPSP synthase enzyme for the generation of glyphosate tolerance *in planta*.

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#### EXAMPLE 6

Immature embryos of wheat (*Triticum aestivum* L) cultivar Bobwhite are isolated from the immature caryopsis 13-15 days after pollination, and cultured on CM4C (Table 3) for 3-4 days. The embryos showing active cell division, but no apparent callus formation are selected for *Agrobacterium* infection.

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Table 3. Supplemental Components in Basal Media<sup>1</sup>

	Components	CM4	CM4C	MMS.2C	MMS0
	2,4-D (mg/l)	0.5	0.5	0.2	--
	Pichloram (mg/l) <sup>2</sup>		2.2	2.2	
20	Maltose (g/l)	40.0	40.0	40.0	40.0
	Glutamine (g/l)		0.5	0.5	
	Magnesium Chloride (g/l)		0.75	0.7	
	Casein Hydrolysate (g/l)		0.1	0.1	
	MES (g/l)		1.95	1.95	1.95
25	Ascorbic Acid (mg/l) <sup>2</sup>		100.0	100.0	100.0
	Gelling Agent (g/l) <sup>3</sup>	2(P)	2(P)	2(G)	2(G)

<sup>1</sup>All media contain basal salts (MS basal salts) and vitamins (MS vitamins) from Murashige and Skoog (1962). The pH in each medium is adjusted to 5.8.

<sup>2</sup>Filter-sterilized and added to the medium after autoclaving.

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<sup>3</sup>Phytigel™ (P) or Gelrite®(G).



A disarmed *Agrobacterium* strain C58 (ABI) harboring a binary vector of interest (pMON45367) is used for all the experiments. The pMON45367 is transferred into *Agrobacterium* by a triparental mating method (Ditta *et al.*, Proc. Natl. Acad. Sci. 77:7347-7351). Liquid cultures of *Agrobacterium* are initiated from glycerol stocks or from a freshly streaked plate and grown overnight at 26°C-28°C with shaking (approximately 150 rpm) to mid-log phase ( $OD_{660}=1-1.5$ ) in liquid LB medium, pH 7.0 containing 50 mg/l kanamycin, 50 mg/l streptomycin and spectinomycin and 25 mg/l chloramphenicol with 200  $\mu$ M acetosyringone (AS). The *Agrobacterium* cells are resuspended in the inoculation medium (liquid CM4C) and the density is adjusted to  $OD_{660}$  of 1. The immature embryos cultured in CM4C medium are transferred into sterile petri plates (16 x 20 mm) or wells of a 6-well cell culture plate (Costar Corporation, Cambridge, MA) containing 10 ml of inoculation medium per petri plate or 5 ml per cell culture cluster plate. An equal amount of the *Agrobacterium* cell suspension is added such that the final concentration of *Agrobacterium* cells is an  $OD_{600}$  of 0.5. In most experiments, pluronic F68 is added to the inoculation mixture at a final concentration of 0.01%. The ratio between the *Agrobacterium* and immature embryos is about 10 ml: 20-200 IEs. The inoculation is allowed to proceed at 23°C-26°C from 5-60 minutes.

After the inoculation period, the remaining *Agrobacterium* cells are removed from the explants by using vacuum aspiration equipment. A piece of sterile Whatman No. 1 filter paper (to fit the size of the petri plate) is placed in each of 60 x 15 or 60 x 20 mm petri dishes. Two hundred  $\mu$ l of sterile water is placed in the middle of the filter paper. After 2-3 minutes, the inoculated immature embryos are placed in the plates. Usually, 20-

50 explants are grouped as one stack (about 1 cm in size and 60-80 mg/stack), with 4-5 stacks on each plate. The plates are immediately covered with Parafilm® and then co-cultivated in the dark at 24°C-26°C for 2-3 days.

The co-cultivated PCIEs are transferred CM4C+500 mg/l carbenicillin medium (delay medium) at dark. After 7 days on the delay medium, the immature embryos are transferred to CM4C supplemented with 2 mM glyphosate and 500 mg/l carbenicillin for selection for one week. Then calli are transferred to MMS0.2C+0.1mM glyphosate +250 mg/l carbenicillin medium for 2 weeks under light for further selection. Embryogenic calli are transferred to a second regeneration medium MMS0C with lower glyphosate concentration (0.02 mM) and 500 mg/L carbenicillin for plant regeneration. Those embryogenic calli are transferred onto fresh medium every two weeks. Regenerated plantlets are transferred to Sundae cups (Sweetheart Cup Company, Chicago, IL) containing the second regeneration medium for further growth and selection. When roots are well established from transgenic plants the plants are transferred to soil for further evaluation.

#### EXAMPLE 7

Novel glyphosate-resistant EPSP synthases can be designed based on the *E. indica* glyphosate resistant EPSPS. The amino acid sequence deduced from the cDNA sequence shows that two amino acid substitutions distinguish the mature EPSP protein sequence derived from the glyphosate-tolerant *E. indica* biotype (top row, Figure 9) from that of the glyphosate sensitive *E. indica* biotype EPSPS protein sequence (bottom row, Figure 9). The substitution of a serine for a proline at position 107 of the *E. indica* EPSPS amino acid sequence and in the corresponding amino acid position in both higher plant and

bacterial EPSP synthase enzymes is known to result in the enzyme having resistance to glyphosate (Padgett *et al.*, J. Biol. Chem. 266:22364-22369 (1991); U.S. Patent No. 4,535,060). All catalytic domain single amino acid substitution EPSP synthase variants characterized to date that exhibit increased tolerance to glyphosate have a higher  $K_i$  for

5 glyphosate, but also have an increase in the apparent  $K_m$  for PEP and reduced  $V_{max}$ , thereby lowering the catalytic efficiency ( $V_{max}/K_m$ ) of the enzyme (Kishore *et al.*, Annu. Rev. Biochem. 57:627-663 (1988); glyphosate (Padgett *et al.*, J. Biol. Chem. 266:22364-22369 (1991). In contrast, the *E. indica* glyphosate resistant EPSP synthase (*E. indica* glypR) exhibits a high affinity for PEP, while retaining significant catalytic efficiency in the

10 presence of glyphosate (TABLE 4). The engineered petunia (*Petunia hybrida*) and corn (*Zea mays*) glyphosate resistant (glypR) variants that in studies by others (U.S. Patent No. 5,866,774; U.S. Patent No. 6,040,497) have shown to confer a high level of glyphosate tolerance in transgenic plants were assayed for affinity for PEP and inhibition by

glyphosate. All of the naturally occurring wild type (wt) *Z. mays* glyphosate sensitive (*Z. mays* glypS), wild type *P. hybrida* glyphosate sensitive (*P. hybrida* glypS) and the *E. indica* glypS and *E. indica* glypR wild type EPSPS enzymes have very similar  $K_m$  values for PEP. The single amino acid substitutions engineered into the catalytic domain of *P. hybrida* EPSPS enzyme drastically increases the  $K_m$  for PEP. The single amino acid

15 substitution found in this domain in the naturally occurring variant of *E. indica* glypR was found to not have a major effect on the  $K_m$  indicating that this enzyme will continue to

20 function well in the plant chloroplast. It required a double mutation in the *Z. mays* EPSPS enzyme to achieve a low  $K_m$  for PEP.

Table 4. Comparison of the apparent  $K_m$  for PEP and apparent  $K_i$  for glyphosate of the *E. indica* glyphosate resistant EPSPS with other known plant EPSPS modified for glyphosate resistance.

	<u>EPSPS enzyme</u>	<u><math>K_m</math> PEP (<math>\mu</math>M)</u>	<u><math>K_i</math> Glyphosate (<math>\mu</math>M)</u>
5	<i>E. indica</i> glypS	5	0.05
	<i>E. indica</i> glypR (Pro-Ser)	7	1
	<i>Z. mays</i> glypS (wt)	5	0.2
	<i>Z. mays</i> glypR (Thr-Ile, Pro-Ser)	5	60
10	<i>P. hybrida</i> glypS (wt)	5	0.4
	<i>P. hybrida</i> glypR (Pro-Ser)	44	3
	<i>P. hybrida</i> glypR (Gly-Ala)	200	2000
	<i>P. hybrida</i> glypR (Gly-Ala, Pro-Ser)	340	8500

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$K_m$ (PEP) determinations for the different enzymes are performed at saturating shikimate-3-phosphate (S3P) concentrations, which is determined according to standard methods (Fersht, *Enzyme Structure and Mechanism*, W.H. Freeman and Co., Ltd., San Francisco, CA., 1977). A series of PEP concentrations are tested, such that the final

20 range of concentrations spans one order of magnitude above and below the experimentally determined  $K_m$ .  $K_i$  (glyphosate), with respect to PEP, is determined in a similar manner, at saturating S3P concentrations, except that velocity vs. [PEP] is determined for a range of glyphosate concentrations (Orsi in "Methods in Enzymology" (Purich, ed.), vol. 63, pg. 159-183, 1979). Calculations, graphical representation, and statistical analysis of enzyme

25 kinetic data are performed using GraFit version 3.0 software (Erithacus Software Ltd., Staines, U.K.).

It is anticipated by this study that transgenic plants resistant to glyphosate are made by transformation with an *E. indica* glyphosate resistant EPSPS gene construct that can include additional modification of the naturally occurring amino acid sequence. These

30 changes are to be made by site-directed mutagenesis of the codons of DNA sequence to

incorporate other known amino acid substitutions in glyphosate resistant plant EPSPSs, such as the threonine to isoleucine substitution at 103 (U.S. Patent No. 6,040,497), and glycine to alanine substitution at 102 (U.S. Patent No. 5,188,642) in the catalytic domain of the *E. indica* EPSPS amino acid sequence. Furthermore, it is anticipated that the catalytic domain of the *E. indica* EPSPS, as well as other plant EPSP Synthases, can be modified to the amino acid sequence of the catalytic domain of the *Agrobacterium* strain CP4 glyphosate resistant EPSPS (U.S. Patent No. 5,633,435) by the same methods. This modification will result in the plant derived EPSPS possessing similar PEP binding and glyphosate resistance as the CP4 glyphosate resistant EPSPS that has been used in cotton, corn, canola, soybeans, potato, wheat, sugarbeet and other agronomically important crop plant to impart plant tolerance to glyphosate. Modification of plant EPSP Synthases to the CP4 EPSPS catalytic domain sequence may comprise the deletion of an amino acid and the substitution of other amino acids. In particular the deletion of the amino acid at 107 of the *E. indica* EPSPS sequence (Figure 2) or the same relative amino acid position in other plant EPSP Synthases that can in addition to the deletion, include substitutions of an alanine for a glycine at 102, glycine for alanine at 104, cysteine for methionine at 105, methionine for alanine at 110, or glycine for alanine at 111. Previous random and site-directed mutations in the conserved region (catalytic domain) of bacterial and plant EPSPSs have shown that modifications that increase the  $K_i$  for glyphosate while keeping the  $K_m$  for PEP low are important for an enzyme that is useful for genetically modifying plants for glyphosate tolerance (U.S. Patent No. 5,866,775).

#### EXAMPLE 8

*E. indica* EPSPS regulatory sequences can be isolated by any number of methods known to those of skill in the art for genomic library preparation. For genomic libraries of the present invention, *E. indica* genomic DNA is isolated by a CsCl purification protocol according to Current Protocols in Molecular Biology, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates); or by a CTAB purification method (Rogers *et al.*, Plant Mol. Biol., 5:69, 1988). Reagents are available commercially (see, for example Sigma Chemical Co., St. Louis, MO). The genomic DNA libraries are prepared according to manufacturer instructions (Genome Walker™, CloneTech Laboratories, Inc, Palo Alto, CA). In separate reactions, genomic DNA is subjected to restriction enzyme digestion overnight at 37°C with the following blunt-end endonucleases: *EcoRV*, *Sca1*, *Dra1*, *PvuII*, or *Stu1* (CloneTech Laboratories, Inc. Palo Alto, CA). The reaction mixtures are extracted with phenol:chloroform, ethanol precipitated, and resuspended in Tris-EDTA buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA). The purified blunt-ended genomic DNA fragments are then ligated to the Genome Walker™ adapters and ligation of the resulting DNA fragments to adapters were done according to the manufacturer's protocol. After ligation, each reaction is heated treated (70°C for 5 min) to terminate the reaction and then diluted 10-fold in Tris-EDTA buffer. One µl of each respective ligation is then amplified in a 50 µl reaction according to manufacturer's recommended protocol using an adaptor-specific oligonucleotide (supplied by manufacturer) and an *E. indica* EPSP synthase gene-specific oligonucleotide, such as SEQ ID NO 4. One µl of each primary reaction is diluted 50-fold and 1 µl of this dilution is then amplified in a secondary amplification using a "nested" adaptor-specific oligonucleotide (supplied by manufacturer) and a "nested" gene-specific oligonucleotide

such as SEQ ID NO 5. PCR products, representing 5' regions of the *E. indica* EPSP synthase gene are then purified by agarose gel electrophoresis using a QIAquick Gel Extraction Kit (cat.# 28704, Qiagen Inc., Valencia, CA) then directly cloned into the pCR2.1-TOPO vector (cat.# K4500-40, Invitrogen, Carlsbad, CA). The identity of the  
 5 cloned PCR products is confirmed by DNA sequence analysis (ABI Prism™ 377, Perkin Elmer, Foster City, CA). The same *E. indica* Genome Walker™ libraries and methods that are used to isolate the *E. indica* EPSP synthase 5' region can be used to isolate the 3' region of *E. indica* EPSP synthase gene, by substituting gene-specific primers (first round SEQ ID NO: 13 and second round SEQ ID NO: 14) that anneal to the 3' end of the gene.  
 10 Amplification products are cloned and verified as for the 5' end of the *E. indica* EPSP synthase gene.

5'-TGCAATCCGGACTGAGCTAACAAAGC-3' (SEQ ID NO: 13)

5'-ACTGCATTATCACACCGCCCGAGAAG-3' (SEQ ID NO: 14)

The translation initiation codon is determined for the *E. indica* EPSP synthase  
 15 gene by inspection, anticipating an initiation codon approximately 63 codons upstream of the start of the mature protein codon region, based on comparison to the maize EPSP synthase gene. Primers are then designed to amplify approximately 2.5 kb of the 5' region beginning at the initiation codon. These primers incorporate restriction sites for cloning into expression vectors, for example, placing a EcoR1 site in the 5' end of promoter  
 20 region the *E. indica* EPSP synthase gene and an Nco1 site incorporating the translation start. Such primers are added in a 50 µl RT-PCR reaction at a final concentration of 25 µM with 50 ng of *E. indica* genomic DNA. PCR amplifications are then performed using the Expand High Fidelity PCR System (cat.# 1 732 641, Roche Molecular Biochemicals,

Indianapolis, IN) per manufacturer's instructions. A thermal profile of 94°C for 30 seconds, followed by 60°C for 30 seconds, then 72°C for 3 minutes is used for thirty cycles. This is followed by a cycle of 72°C for 3 minutes. The gel purified amplification product is then digested with Pst1 and Nco1.

5           The 3' end of the *E. indica* EPSP synthase gene is amplified using two gene specific primers (SEQ ID NO:15 and SEQ ID NO:16) which incorporate a BamH1 site immediately downstream of the translation stop codon and a Pst1 approximately 650 bases downstream of the translation stop codon. The product is amplified as for the 5' end of the *E. indica* EPSP synthase gene except an extension time of 1 minute is used. The gel  
10 purified product is digested with BamH1 and Pst1.

5'-CTAAGGATCCTCTGTGCCTGCCTCATGAAGAGAGTT-3' (SEQ ID NO: 15)

5'- TGATCTGCAGGCAAGTGTCTTACCCTTACCCTTCTG -3' (SEQ ID NO: 16)

          The 5' (EcoR1/Nco1 fragment) regulatory and 3' (BamH1/Pst1 fragment) regulatory regions of the *E. indica* EPSP synthase gene can be ligated to a compatibly  
15 digested vector and a coding region to generate a transgene capable of expressing a transcript under control of *E. indica* EPSP synthase gene regulatory elements. An example of such is a coding region would be the *A. tumefaciens* strain CP4 EPSP synthase gene (U.S. Patent No. 5,633,435) expressed under the control of the *E. indica* regulatory sequences.

20           The basal expression of the *E. indica* EPSP synthase gene promoter may be modified to enhance its expression. Methods known to those of skill in the art can be used to insert enhancing elements (for example, subdomains of the CaMV 35S promoter, Benfey et. al, 1990 EMBO J. 9: 1677-1684) into the *E. indica* EPSP synthase gene 5'



sequence to generate a promoter which encompasses the temporal and spatial expression of the *E. indica* EPSP synthase gene but have quantitatively higher levels of expression. Similarly, tissue specific modifications of the *E. indica* EPSP synthase 5' region expression can be accomplished with elements determined to specifically activate or  
5 repress gene expression (for example, pollen specific elements, Eyal *et al.*, 1995 Plant Cell 7: 373-384).

From the foregoing, it will be seen that this invention is one well adapted to attain all the end and object herein above set forth together with advantages that are obvious and that are inherent to the invention.

10 The embodiments described above are provided to better elucidate the practice of the present invention. Many possible embodiments may be made of the invention without departing from the scope thereof, it should be understood that these embodiments are provided for illustrative purposes only, and are not intended to limit the scope of the invention.

15 It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims. All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and  
20 individually indicated to be incorporated by reference.